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Cytoskeletal Lesions in Anoxic Myocardial Injury

A Conventional and High-Voltage Electron-Microscopic and Immunofluorescence Study

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The role of cell swelling in mediating myocardial injury was studied in control (normoxic) and anoxic Langendorf perfused rat hearts. Control and 45-, 75-, or 90-minute anoxic hearts were exposed to hypotonic (200 mOsm/l) perfusion media to induce osmotic swelling. Anoxic hearts, but not control hearts, released myoglobin when subjected to osmotic swelling. Control hearts, exposed to hypotonic swelling, retained an intact cytoskeletal system of intermediate filaments, microfilaments and microtubules, intact sarcoplasmic reticulum, and intact sarcolemmal membranes. In contrast, swollen anoxic hearts showed a variety of ultrastructural lesions, including formation of large subsarcolemmal blebs associated with lysis of lateral Z-, M-, and A-band intermediate filament attachments, vesiculation of sarcoplasmic reticulum, and rupture of sarcolemmal membranes. Inter-mediate filament attachments with nuclear membranes were also broken, and microtubules disappeared from the perinuclear space. Sarcomere changes included distortion of Z bands, loss of lateral Z band-sarcolemmal attachments, and separations of myofibrils from internal faces in intercalated disks. Immunofluorescence studies of control hearts showed normal staining patterns for the cytoskeletal-associated proteins vinculin, α-actinin, and desmin. After 90 minutes of anoxia, hearts exhibited diminished staining of vinculin and α-actinin and relatively little change in desmin staining. The results demonstrate that a critical period of anoxia causes lesions in the cytoskeletal apparatus of myocardial cells which correlate with the increased osmotic fragility of irreversibly injured anoxic myocardial cells. (Am J Pathol 1987, 129:327–344)

IRREVERSIBLE injury can be defined as the inability of cells to remain viable when the injurious agent is removed and the cells are returned to a normal environment. In adult myocardium a prolonged period of oxygen and substrate depletion renders hearts susceptible to severe cellular damage when reexposed to oxygen. Upon reoxygenation the cells swell explosively with the formation of subsarcolemmal blebs, sarcomeres hypercontract and form contraction bands, and plasma membranes rupture, allowing release of soluble cytoplasmic proteins to the extracellular space and the admission of extracellular calcium into the cytosolic space.[1-3] It has recently been questioned whether the lethal cell injury occurs during the initial anoxic interval or at the time of reoxygenation. In the present study we have attempted to determine whether latent injury occurs in anoxic cells which can account for loss of sarcolemmal membrane integrity and subsequent cell death in the absence of reoxygenation. We have postulated that the lesions of irreversible injury are latent and become apparent only when cells are exposed to the physical stresses of contracture and/or swelling that occur at the time of reperfusion. This hypothesis requires that anoxic cells have inapparent, but experimentally demonstrable, lesions that could account for loss of plasma membrane integrity upon reperfusion in the absence of reoxygenation. There is evidence that such latent lesions occur in irreversibly injured myocardium.

Cellular swelling can occur when an osmotic gra-

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dient is applied across cell membranes. This can occur when cells adapted to a hypertonic medium are suddenly exposed to isotonic media or when normal cells are directly exposed to hypotonic media. Normal cells, however, do not exhibit ideal osmotic behavior and actually swell less than would be predicted when exposed to a given osmotic gradient. The resistance of cells to swelling may imply that there are mechanical restraints which resist the osmotic forces until the cell can compensate by reequilibration to its new environment. In ischemic myocardium the reduced blood flow allows retention of metabolic breakdown products so that the tissue becomes hypertonic relative to the extracellular space. Reperfusion with isotonic fluid would suddenly expose the hypertonic cells to an osmotic gradient to induce swelling. Reperfusion of globally ischemic rat hearts has been shown to cause a sudden loss of myoglobin from the cells in the absence of reoxygenation or contracture of the cells.[4] The loss of myoglobin from cells is a marker for severe damage to the plasma membrane and cell death. The cellular swelling that would occur upon reperfusion of hypertonic ischemic cells with isotonic fluids has been suggested to contribute to the cellular damage that occurs upon reperfusion of ischemically injured myocardium.[4,5] This concept has received experimental support from Steenbergen and Jennings,[6] who reported that anoxic slices of myocardium *in vitro* both swell and develop enlarged inulin spaces when exposed to hypotonic media, whereas oxygenated slices resisted osmotic swelling and maintained small inulin spaces under similar conditions. These authors suggested that damage to the sarcolemmal membrane or its underlying cytoskeletal supports may be responsible for the events observed during osmotic swelling.

We have recently found that irreversibly injured anoxic perfused rat hearts demonstrate a parallel susceptibility to reoxygenation injury (oxygen paradox) and the cell injury that results from either passive ventricular distension with an intraventricular balloon or osmotic cellular swelling of anoxic hearts.[7] These studies that irreversibly injured cells become fragile and less capable than normal cells of withstanding the mechanical stresses of stretching or swelling. Because the ability of cells to withstand physical stress depends in part on an intact cytoskeletal system, the present study was initiated to determine whether lesions occur in the cytoskeleton of anoxic myocytes that could account for their increased fragility. The results of this investigation demonstrate that lesions do develop in the cytoskeletal structure of irreversibly injured myocytes, which could contribute to their abnormal responses to osmotic swelling and other physical stresses.

Materials and Methods

Heart Perfusion

Male Sprague–Dawley rats weighing 250–300 g were anesthesized with sodium pentobarbital (Diabutal, Diamond Laboratories Inc., Des Moines, Iowa). After intravenous administration of 2000 IU sodium heparin (Elkins–Sinn, Cherry Hill, NJ), hearts were removed and rapidly immersed in ice-cold Krebs-Henseleit bicarbonate (KHB) solution. After trimming and weighing, hearts were mounted on a triple reservoir nonrecirculating Langendorf apparatus and perfused at 85 mm Hg pressure. Temperature was maintained at 37 ± 0.5 C.

Control perfusion media (KHB) contained 126 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.8 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 11 mM glucose and had a final osmolality of 300 mOsm/l. Anoxic buffer was prepared in a similar manner except 11 mM mannitol was substituted for glucose. Hypotonic media was prepared by dilution of the standard media with deionized, distilled water to a final osmolality of 200 mOsm/l. Media was equilibrated and with 95% O_2–5% CO_2 (normoxic) or 95% N_2–5% CO_2 (anoxic), adjusted to a pH of 7.35–7.40, and continuously gassed for the duration of the experiment with the appropriate gas.

Myoglobin Analysis

Myoglobin concentration of aliquots of effluent collected during the experiments was calculated from adsorbance readings at a wavelength of 414 nm with horse heart myoglobin (Sigma Laboratories, St. Louis, Mo) as standards. Myoglobin release was reported as micrograms per minute per gram initial wet heart weight.

Fixative Solutions

Isotonic fixative was a freshly prepared solution of 1% glutaraldehyde in a modified Tyrode's buffer with a final osmolarity of 315 mOsm/l to which was added 2.0 g/l of tannic acid. This solution was diluted with deionized, distilled water to produce a hypotonic fixative calculated to have an osmolarity of 200 mOsm/l. For immunofluorescence studies, 30 g/l purified paraformaldehyde was dissolved into the final perfusion buffer used in each experiment. This produced a hypertonic fixative of about 970 or 1070 mOsm/l. The pH was adjusted to 7.4 with NaOH.

After primary glutaraldehyde perfusion, slices from Group 1 hearts were immersed in the same hypotonic glutaraldehyde fixative for an additional 1–3 days.

Tissue from Group 2 hearts, fixed by perfusion with hypertonic paraformaldehyde, was immersion-fixed for 1–3 days in the isotonic glutaraldehyde–tannic acid solution. Postfixation of all tissues was with 1% osmium tetraoxide. Following *en bloc* staining in aqueous uranyl acetate, tissue blocks were dehydrated through a graded series of ethanol solutions, propylene oxide, and embedded in Epon 812.

Microscopy

Three to five blocks of tissue from every heart were sectioned at 1 μ, stained with toluidine blue, and examined by light microscopy. For routine transmission electron microscopy, sections from at least two blocks from each heart were cut at either 500 Å (silver) or 900 Å (gold) and mounted on unsupported copper grids. After staining with aqueous uranyl acetate and lead citrate, sections were examined and photographed at 60 kev with JEOL 100 CX electron microscope.

For high-voltage electron microscope (HVEM) studies, two blocks from each of three hearts in each experimental series were sectioned at 0.25 or 0.5 μ and mounted on one-hole formvar-coated grids. After immersion staining of the grids in 50% ethanolic uranyl acetate at 60 C for 3–5 hours, the sections were carbon-coated in a vacuum evaporator. Sections were examined with the AEI microscope located in the High Voltage Electron Microscope Laboratory at the University of Wisconsin (Madison, Wis). Accelerating voltage was 1 MeV, and for stereographic imaging tilt angles ranging from 6 to 32 degrees were used.

Immunofluorescence Studies

Frozen sections of paraformaldehyde-fixed tissues from Group 2 hearts were mounted on glass slides. After brief exposure to 0.1% BSA in phosphate-buffered saline, the sections were stained with the primary antibodies. Monoclonal mouse anti-vinculin and polyclonal rabbit anti-desmin (ICN Immuno-Biologicals, Lisle, Ill) were diluted to 1/40 and 1/20 and allowed to stain the cryostat sections for 2 hours at room temperature. Polyclonal mouse anti-α-actinin was obtained from the same source but was used at a 1/10 dilution, and staining was overnight (12 hours) at room temperature. After primary antibody labeling, appropriate fluorescein-labeled secondary antibodies (Kirkegard and Perry Laboratories Inc., Gaithersburg, Md) were diluted to 1/20 and allowed to react with the sections for 1–2 hours at room temperature. Controls included unfixed fresh heart tissue and 2-hour staining with omission of the primary antibody. Sections were examined with a Leitz epifluorescence microscope. Negative prints were prepared directly from color slides.

Experimental Design

Two groups of experiments were conducted. The first group was designed to provide well-fixed swollen cells for morphologic studies and utilized hypotonic glutaraldehyde fixation. The second group was used for myoglobin release studies, to provide perfusion-fixed tissue for immunofluorescence (Table 1).

Group 1

Hearts were perfused for an initial 15-minute equilibration period. With three hearts for each set of experiments, hearts were perfused for 75 minutes with either anoxic or oxygenated isotonic medium and then for 15 minutes with hypotonic medium. They were then perfusion-fixed for 3–5 minutes with hypotonic glutaraldehyde–tannic acid solution.

Group 2

Hearts were equilibrated for 15 minutes with oxygenated isotonic perfusate. 1) A set of four hearts were then perfused for 60 minutes with oxygenated isotonic medium followed by 15 minutes with hypotonic (200 mOsm) oxygenated medium (controls). 2) A set of eight hearts were subjected to anoxic, isotonic perfusion for 45 minutes. Subsequently, four of these hearts were perfused for 15 minutes with isotonic anoxic medium; the other four were perfused with hypotonic anoxic medium. 3) Hearts were treated as in 2 except that the initial anoxic period was extended to 90 minutes. All Group 2 hearts were perfusion-fixed with hypertonic paraformaldehyde fixative. After fixation, each heart was divided for use either for frozen sections or was further immersion-fixed in isotonic glutaraldehyde-tannic acid. Effluents of Group 2 hearts were used for myoglobin release studies.

Table 1—Experimental Design

Group 1		
Control, O_2, 75 min	Hypotonic O_2, 15 min	Hypotonic fixation
Anoxic, N_2, 75 min	Hypotonic N_2, 15 min	Hypotonic fixation
Group 2		
Control, O_2, 60 min	Hypotonic O_2, 15 min	Hypertonic fixation
Anoxic, N_2, 45 or 90 min	Hypotonic N_2, 15 min	Hypertonic fixation
	Isotonic N_2, 15 min	

Statistics: All results are reported as the mean ± SEM. The student *t* test was used to determine statistical significance with $P < 0.05$ considered significant.

Results

Myoglobin Release

Oxygenated hearts perfused with 300 mOsm/l (isotonic) media for 60 minutes released no myoglobin when the perfusate was switched to a 200 mOsm/l (hypotonic) medium (Figure 1).

Anoxic hearts perfused with isotonic medium began a slowly rising release of myoglobin at about 60 minutes, which was sustained for the duration of anoxic perfusion. Switching perfusion buffers to a similar isotonic medium did not affect the rate of myoglobin release. In contrast to control hearts, when anoxic hearts were subjected to hypotonic perfusion, there was a sudden peak of myoglobin release. The peak of myoglobin release after 90 minutes was significantly larger than the peak observed after 45 minutes (Figure 1). The total myoglobin release calculated from the areas beneath the curves was 94.85 ± 46.88 μg/g wet weight for control hearts, 346.04 ± 5.5 μg for 45-minute anoxic hearts ($P < 0.025$ versus control) and 856.86 ± 160.27 μg/g wet weight for 90-minute hearts ($P < 0.025$ versus 45-minute hearts).

Light Microscopy

Oxygenated hearts from Group 1, which were subjected to hypotonic swelling and hypotonic fixation, appeared paler and more swollen than oxygenated hearts from Group 2, which were perfused in the same manner but which were fixed in hypertonic fixative. Otherwise, control hearts contained uniform cells with regular relaxed sarcomere alignments and evenly dispersed nuclear chromatin (Figure 2a and b).

Anoxic Group 2 hearts varied in appearance depending upon whether or not the cells were subjected to hypotonic media prior to fixation. In hearts perfused with isotonic media some cells appeared slightly swollen, and nearly all exhibited contraction of sarcomeres and prominent clumping of nuclear chromatin. These changes were more severe at 90 minutes, compared with 45 minutes, and swelling was more obvious and uniform in most 90-minute cells. In addition, many cells in 90-minute anoxic hearts showed an apparent separation and pulling away of sarcomeres from intercalated disk junctions (Figure 2c and e). A few swollen cells contained small subsarcolemmal blebs.

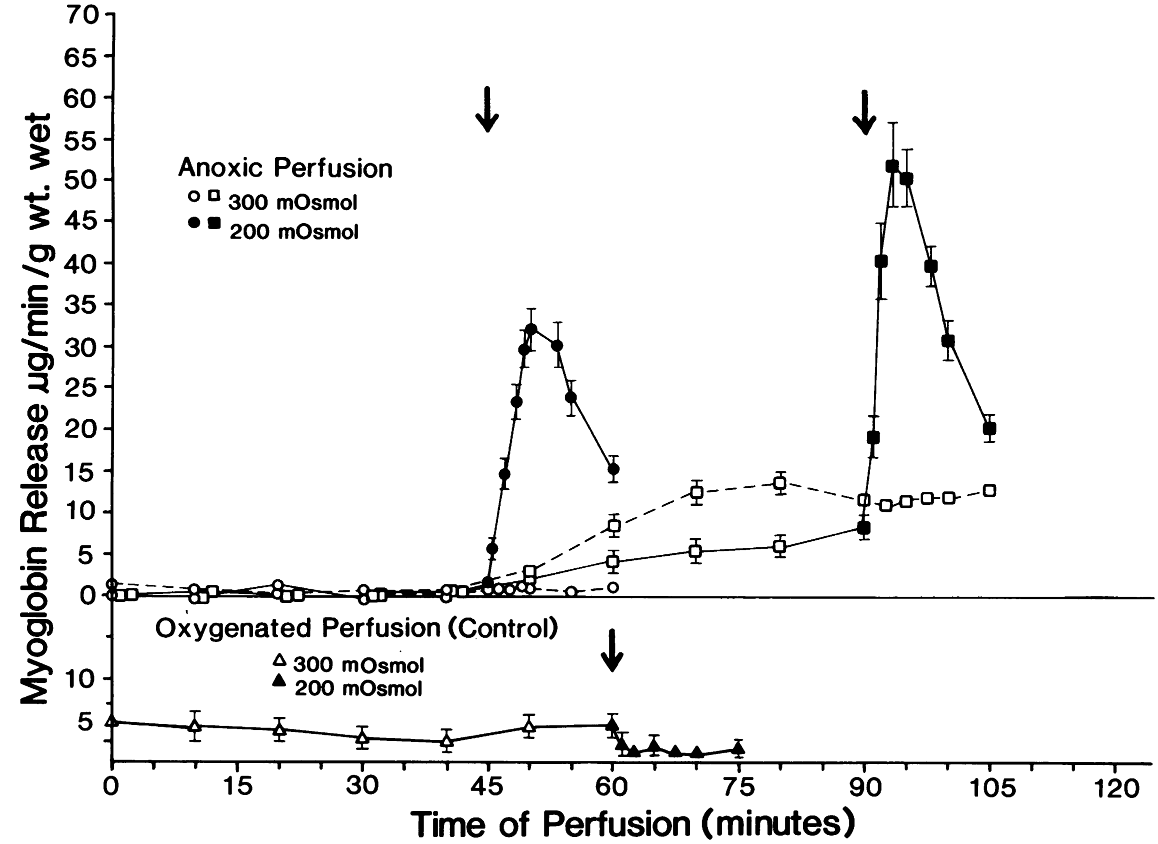

Figure 1—Myoglobin release curves from anoxic and oxygenated-perfused rat hearts. Anoxic hearts exposed to isotonic (300 mOsm/l) medium began a slowly rising myoglobin release after about 60 minutes of perfusion. Exposure of anoxic hearts to hypotonic (200 mOsm/l) medium at 45 or 90 minutes resulted in sudden peaks of myoglobin release. Control oxygenated hearts (below) released no myoglobin during either isotonic perfusion or after hypotonic perfusion. Each point represents the mean ± SEM for four hearts.

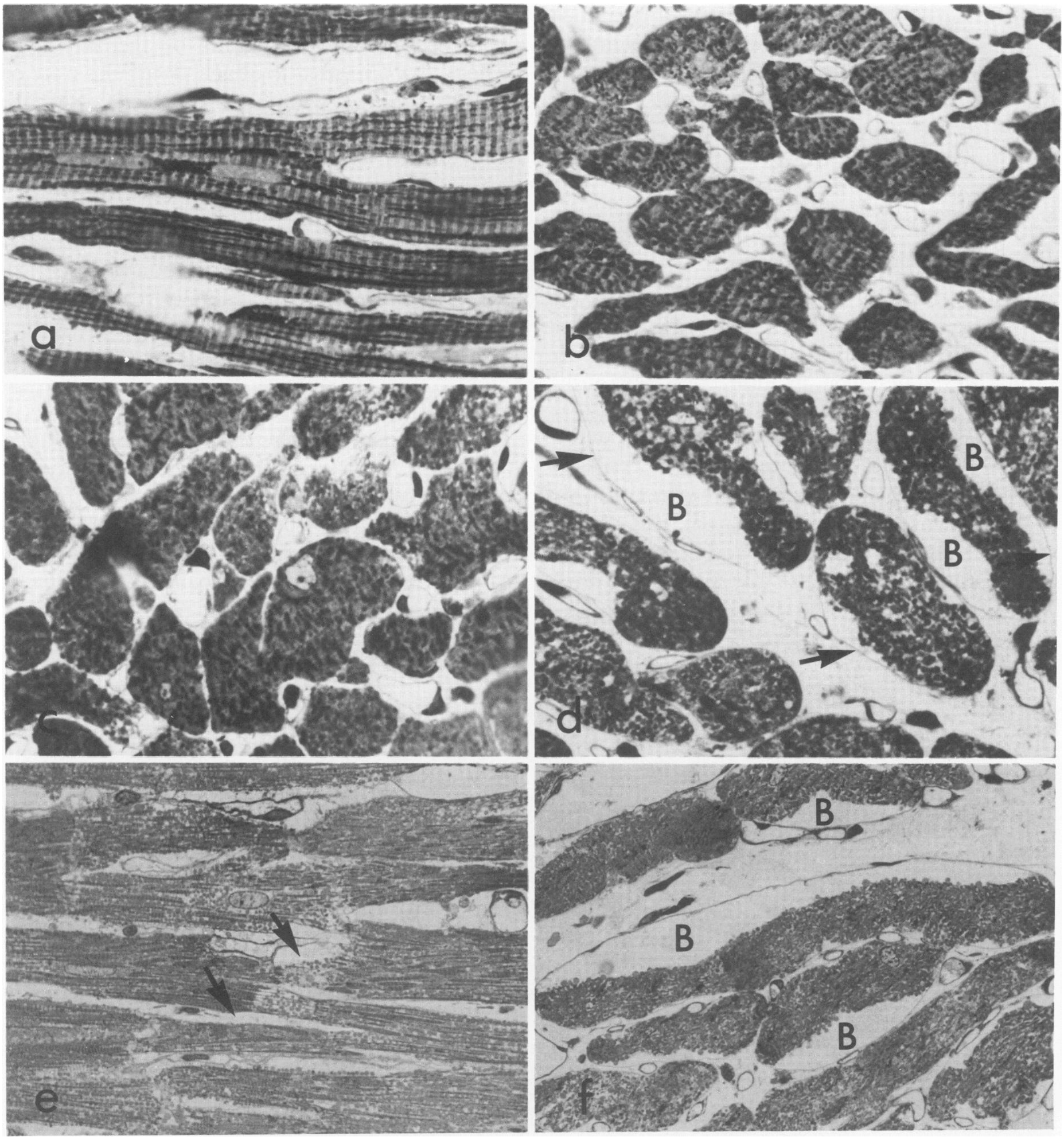

Figure 2a and b—Sections of control oxygenated hearts perfused for 60 minutes with isotonic medium and then by 15 minutes with hypotonic medium. **c** and **d**—Forty-five minute anoxic hearts subjected to either 15 minutes of isotonic perfusion (**c**) or 15 minutes of hypotonic perfusion (**d**). Cells perfused with isotonic medium appeared similar to controls. After hypotonic swelling, injured myocytes developed large subsarcolemmal blebs (*B* and *arrows*). **e** and **f**—Ninety-minute anoxic hearts subjected to 15 minutes of isotonic (**e**) or hypotonic (**f**) perfusion. After isotonic anoxia (**e**), cells appear slightly swollen and sarcomeres are retracted from intercalated disks (*arrows*). Few subsarcolemmal blebs are evident. Anoxic cells swollen in hypotonic medium (**f**) developed large subsarcolemmal blebs (*B*). All hearts were from Group 2 and were fixed with hypertonic paraformaldehyde (**a–d**, ×300; **e** and **f**, ×200)

Cells from anoxic hearts (Group 2) subjected to hypotonic swelling showed prominent blebs beneath the sarcolemma. Approximately half of the cell population in 45-minute hearts and most cells in 90-minute hearts exhibited sarcolemmal blebs (Figure 2d and e). In addition, small groups of cells contained contraction bands.

The changes in injured cells of anoxic Group 1 hearts were indistinguishable from those in Group 2 hearts, indicating that the choice of a hypertonic or hypotonic fixative did not alter the final appearance of injured cells or the size of the subsarcolemmal blebs. The apparently noninjured population of cells, however, appeared more swollen in Group 1 than in Group 2 hearts.

Electron Microscopy

Oxygenated Hearts

Control oxygenated hearts in both Group 1 and Group 2 were subjected to hypotonic perfusion prior to fixation. Cells from Group 1 hearts subjected to hypotonic glutaraldehyde fixation were obviously swollen, while the Group 2 hearts subjected to hypertonic fixation contained cells with no apparent swelling (Figures 3 and 4). The swelling of Group 1 hearts revealed details of structure not apparent in nonswollen hearts. In swollen cells, relaxed sarcomeres were in exact lateral register. Cables of filaments extended across the interfibrillar cytoplasmic space at the level of the Z bands. The cables extended across the cell to the costamere invaginations of the sarcolemma, where they appeared to attach. T-tubules were often closely associated with these Z-band cables (Figure 3). The mitochondria remained dense and were irregularly shaped, but most had focal lucent areas which opened to a space surrounded by membraneous protrusions. In 500-Å sections there was a large perinuclear space that contained fragments of filamentous material. Although focal vesiculation was present, the sarcoplasmic reticulum generally retained a reliculated arrangement. In both swollen Group 1 and nonswollen Group 2 hearts, the intercalated disks remained intact (Figure 5).

High magnifications showed that the cables at Z and M bands were composed of filament bundles of about 100 Å-thick fibers. At the lateral invaginations of the sarcolemma, the cables appeared to fan out to form lateral attachments to the inner surface of the plasma membrane (Figure 6). Similar but smaller cables eminated from the M band to attach near the

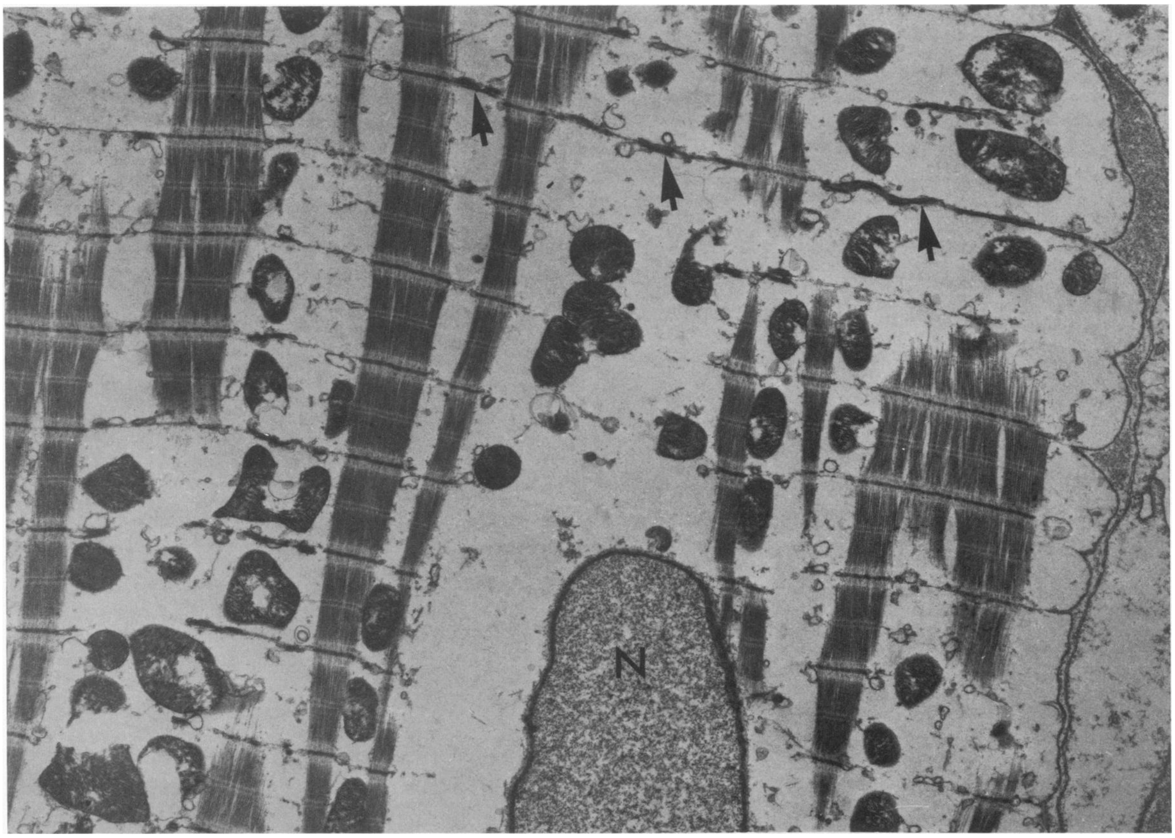

Figure 3—Electron micrograph of a swollen oxygenated Group 1 heart. Separation of organelles allows visualization of cables of intermediate filaments extending across the cell at the level of the Z band. T-tubules are closely associated with the Z-band (*arrows*). The sarcolemma is scalloped with invaginations at the Z-band cable attachments. The nuclear chromatin is evenly dispersed (*N*). Mitochondria have empty cleared spaces. The perinuclear zone is enlarged and contains a few filamentous fragments. (Hypotonic glutaraldehyde fixation, TEM 60 keV, 500-Å section, ×6,800)

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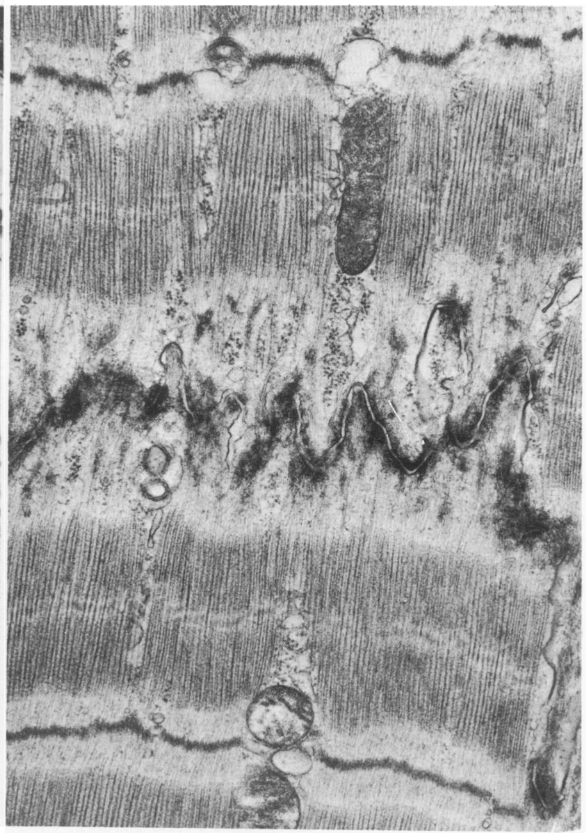

Figure 4—Oxygenated Group 2 hearts subjected to hypertonic fixation after cell swelling. The cells appear similar to normal myocardium. (TEM, 60 keV, 900-Å section, ×5600) **Figure 5**—Oxygenated Group 2 heart. Intercalated disks were intact in control hearts. (TEM, 60 keV, 900-Å section, ×16,400)

dome of the sarcolemmal membrane scallops. At times these M-band cables produced secondary invaginations of the sarcolemma. Rarely, a third set of filaments could be seen extending from the A band to the sarcolemma (see Figure 8).

Examination of 0.25- or 0.5-μ sections with HVEM revealed the perinuclear space of swollen cells to be crossed by intermediate filament cables corresponding to those described at the sarcolemma (Figure 7). Numerous microtubules crossed the space at various angles and appeared to have attachments to both the nuclear membrane and the intermediate filament cables. The nuclear membrane appeared to be tented outward at the attachments of the cables and to be covered by a fuzzy coat of low-density material (Figure 8).

Anoxic Hearts

Injured cells in both Group 1 and 2 hears appeared similar at each time interval. The differences between hearts was largely limited to the relative number of injured cells and the extent of the lesions. Each anoxic heart exposed to hypotonic perfusion contained large numbers of cells with prominent subsarcolemmal blebs (Figure 9). In the area of the bleb, the sarcolemmal membrane lost its scalloped pattern, appeared lifted away from the underlying sarcomeres and its connections to Z-, M- and A-band cables of intermediate filaments appeared broken. In some areas fragments and tufts of material were adherent to the inner surface of the bleb, while in other areas the membrane appeared free of adherent material. At the margins of blebs, T-tubules appeared stretched out, and the yoke of the initial inverted Y-shaped branch of the T-tubules appeared lifted away from the underlying Z band (Figure 10). Focally, there was vesiculation and fragmentation of the sarcolemmal membranes overlying the blebs (Figure 11). Near the centers of large blebs, very elongated, attenuated T-tubules crossed the bleb space. The sarcoplasmic reticulum was extensively vesiculated throughout the cell cytoplasm.

Cells that contained subsarcolemmal blebs also showed loss of intermediate filament attachments to the nuclear membrane (Figure 12). In such cells mi-

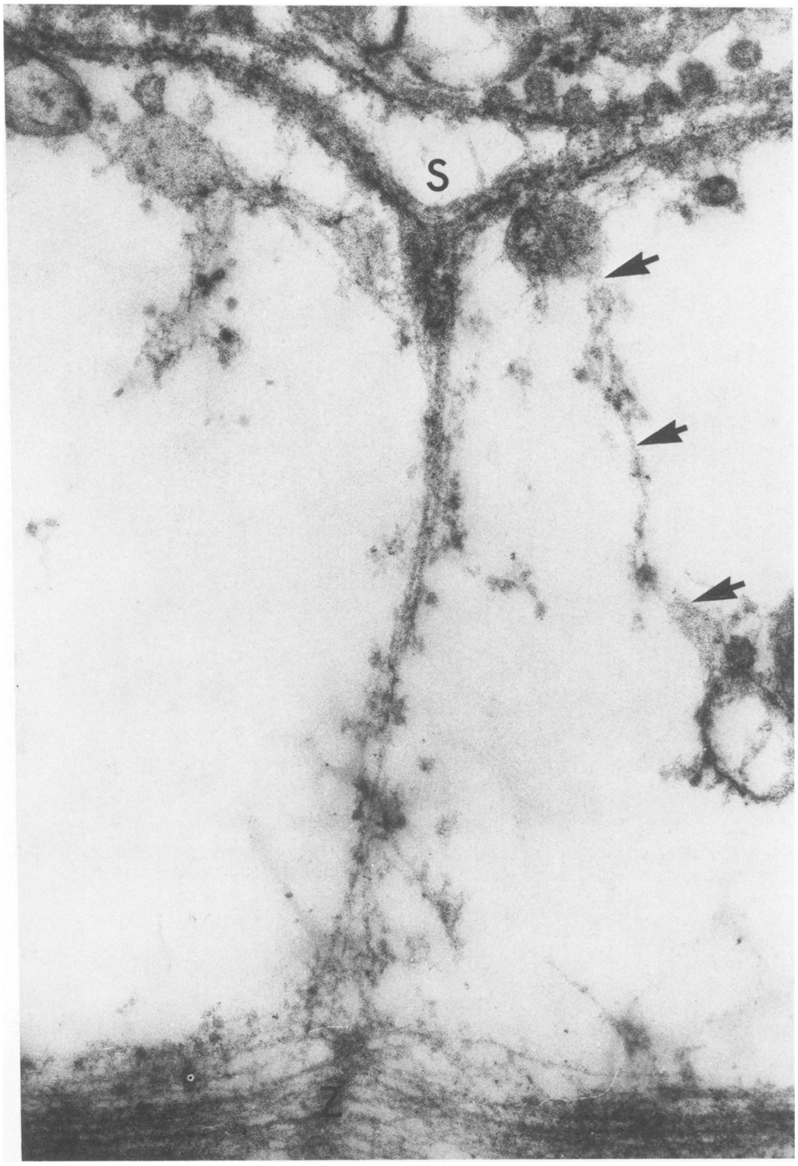

Figure 6—Oxygenated Group 1 heart. A cable of intermediate filaments extends from the invaginated scallop of the sarcolemma (*S*) to the Z band of a myofiber (*Z*). Other intermediate filaments appear to extend between vesicles in the subsarcolemmal space (*arrows*). (TEM, 60 keV, 900-Å section, ×70,200)

crotubules were usually absent or diminished in number from the perinuclear space (Figures 12 and 13). The sarcomeres were sometimes irregularly contracted or stretched with frequent dislocations of Z bands and retractions of sarcomere attachments to the intercalated disks (Figure 14).

Immunofluorescence Studies

In control hearts, vinculin antibodies localized at intercalated disks and periodically along the circumference of the sarcolemmal membrane (Figure 15a). Alpha-actinin showed a similar but less intense localization to intercalated disks but was also localized in a Z-band pattern through the cytoplasm (Figure 16a). Desmin had a periodic staining pattern similar to α-actinin, but the bands of intense staining were broader, and there was no intensification of staining at intercalated disks (Figure 17a).

In anoxic hearts, focal alterations in staining were observed in nonswollen hearts at 45 minutes, which were more uniform and diffuse after 90 minutes. Both vinculin and α-actinin showed a diffuse decrease or loss of staining intensity at intercalated disks, at sarcolemmal membranes, and along Z bands (Figures 15b and 16b). Desmin showed little change in staining

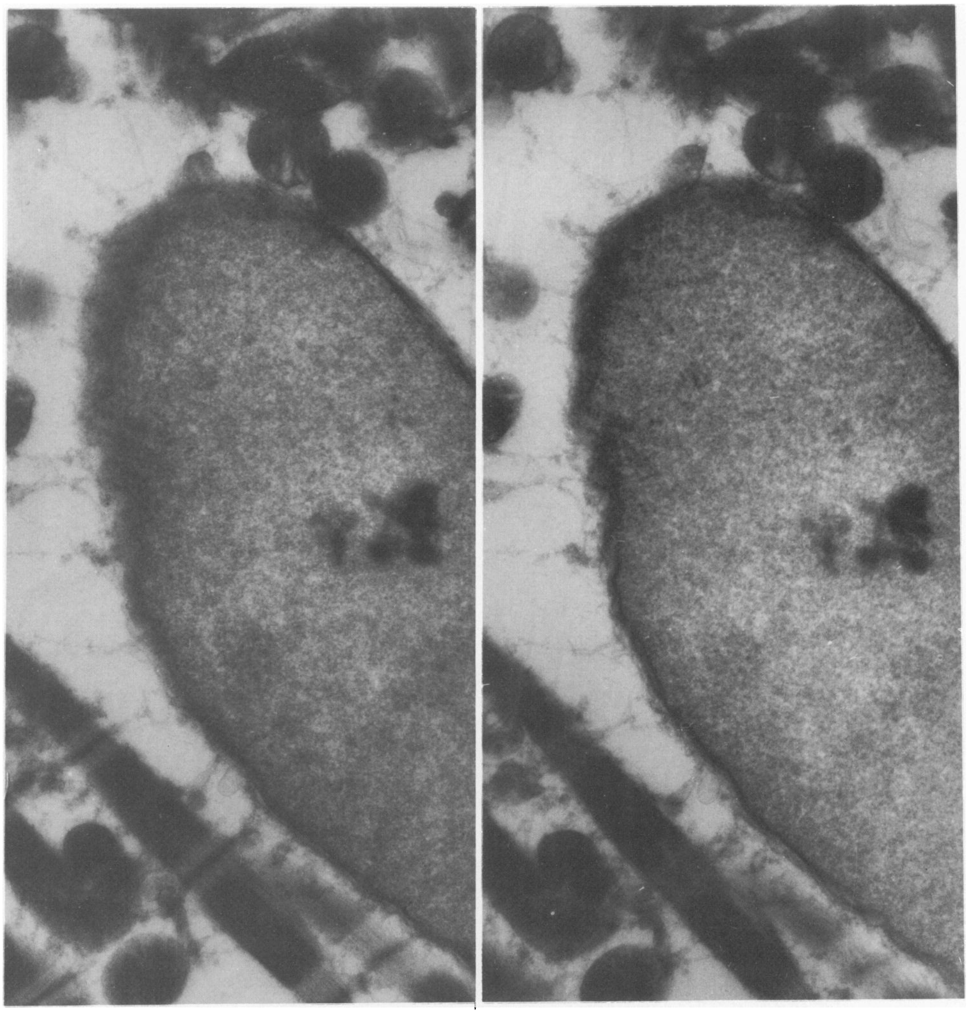

Figure 7—Oxygenated Group 1 heart. The swollen, enlarged perinuclear space contains numerous filaments radiating from the nucleus to the cytoplasm. (HVEM, 1 MeK, 0.5-μ section, stereographic pair, 30° tilt, ×12,500)

pattern or intensity after 45 minutes of anoxia, but by 90 minutes the staining was less uniform throughout the sections (Figure 17b). However, many individual cells were similar to controls.

Excised hearts, which were immediately frozen and stained without aldehyde fixation, showed a staining similar in pattern and intensity to the perfused control hearts. Control sections stained only with secondary antibody showed either no or only diffuse staining with no specific localization.

Discussion

The cytoskeleton of cardiac muscle cells consists of several types of filaments and their associated binding and attachment proteins.[9,10] In adult cardiac muscle, the 10-nm intermediate filaments are composed of desmin.[11-14] Microtubules (25 nm) comprise another type of filamentous structure of the cytoskeleton.[15] In cardiac muscle there are no true stress fibers; their role is assumed by the highly organized sarcomeres forming the contractile elements of the cell. In cardiac cells, the actin filaments form attachments to the cell membrane at the fascia adherens junctions of the intercalated disk and at the lateral costamere junctions,[16] where the sarcolemma makes periodic contact with Z bands. These actin attachment sites contain vinculin, a protein localized internally and presumably bound to the sarcolemma.[17] Recently it has been recognized that vinculin does not directly bind actin, but is a member of a group of proteins that form the attachment sites.[18] The Z bands are composed of overlapping branched ends of actin thin filaments.[19] Alpha-actinin is an actin-binding protein which cross-links actin and forms much of the dense component of both the Z band and fascia adherens plaques.[20]

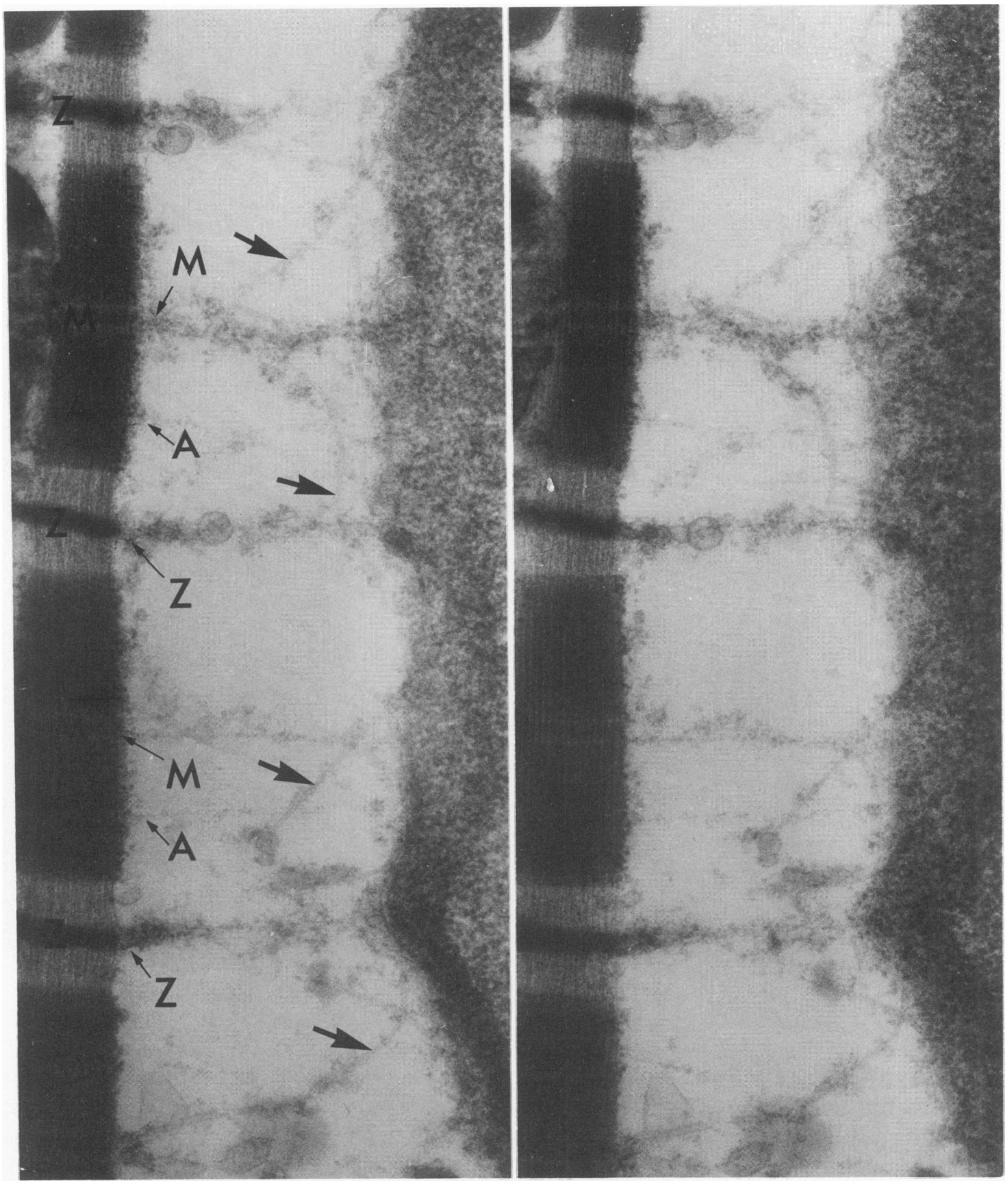

Figure 8—Oxygenated Group 1 heart. Filaments extend across the perinuclear space at the levels of Z, M, and A bands. Numerous microtubules bend at various angles near and in contact with the nucleus (*arrows*). There is outward tenting of the nuclear membrane in areas of Z- and M-band cable contacts. (HVEM, 1 MeV, 0.5-μ section, stereographic pair, 24° tilt, ×35,000)

Intermediate filaments form a transverse cable system that links Z bands of adjacent myofibrils, ultimately attaching to the sarcolemmal membranes at costamere junctions.[21] Desmin also forms a network of transverse and longitudinal fibers in the interfibrillar spaces. Intermediate filaments form lateral attachments to the macula adherens junctions at intercalated disks and also attach to the outer nuclear membrane.[9] In addition, mitochondria and T-tubules apear to be attached to the intermediate filament network.[8] The exact nature of these latter attachments is not known. Microtubules attach to a variety of cytoplasmic elements via a large group of microtubular associated proteins (MAPs).[8] There is a constant association of microtubules with the outer nuclear membrane, and they course for long distances at various angles across the cytoplasmic space.[15] In addition to the cytoplasmic filamentous elements,

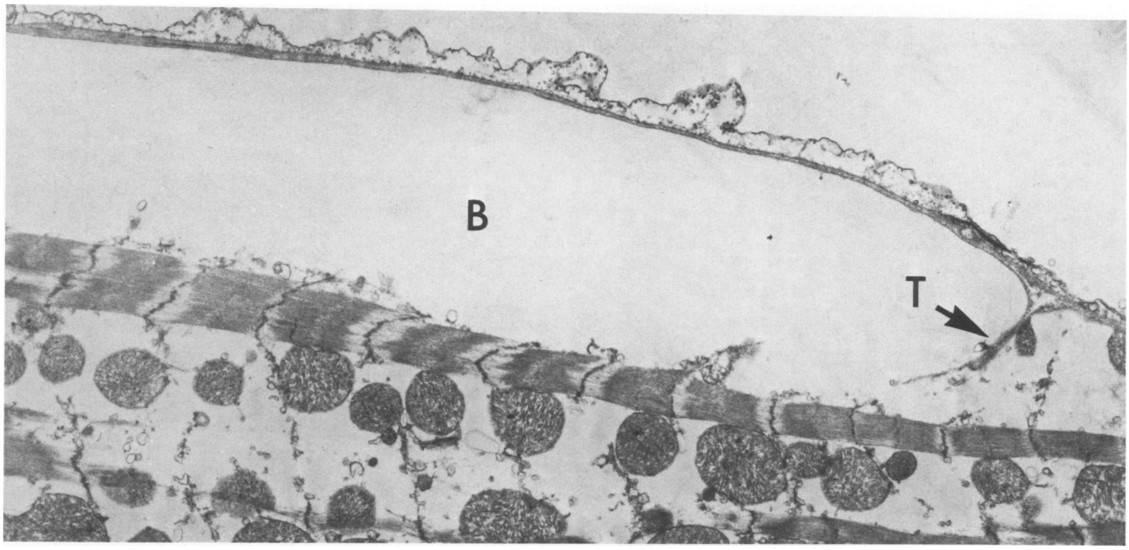

Figure 9—Seventy-five-minute anoxic Group 1 heart. Swollen anoxic cells developed large subsarcolemmal blebs (*B*). The overlying sarcolemma is no longer scalloped, and attachments to Z- and M-band intermediate filament cables are broken. A T-tubule invagination is present near the margin of the bleb (*T*). (TEM, 60 keV, 500-Å section, ×6750)

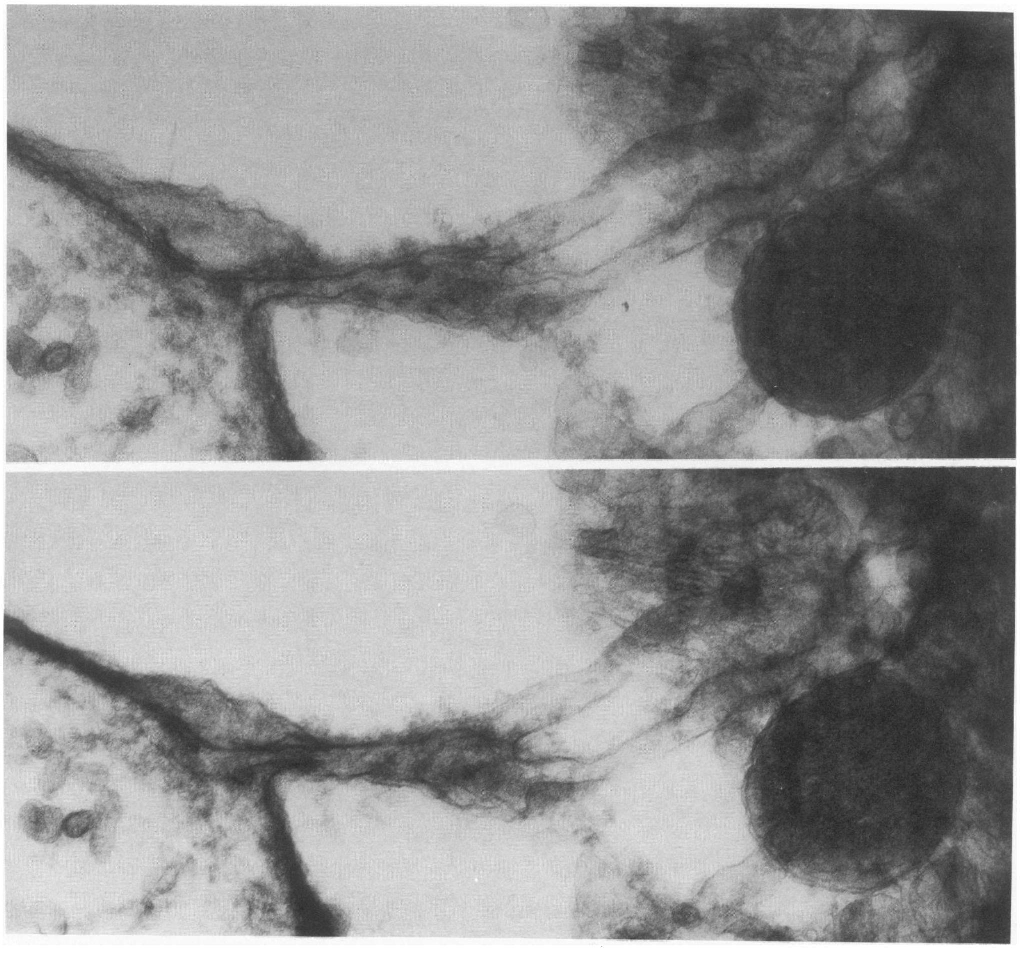

Figure 10—Ninety-minute anoxic swollen Group 2 heart. At a bleb margin a T-tubule crosses the bleb space and branches. The yoke of the branchis pulled away from the underlying sarcomere. (HVEM, 1 MeV, 0.5-μ section, stereographic pair, 31° tilt, ×16,000)

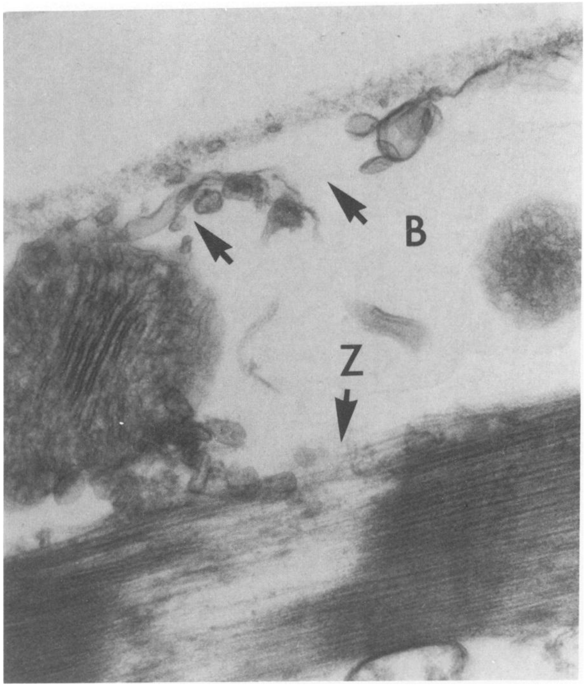

Figure 11—Seventy-five-minute anoxic swollen Group 1 heart. The sarcolemmal membrane over a bleb (*B*) is focally broken and vesiculated (*arrows*). The Z band (*arrow*) of the underlying sarcomere is distorted and appears to be shredding apart. (HVEM, 1 MeV, 0.25-μ section, ×14,400)

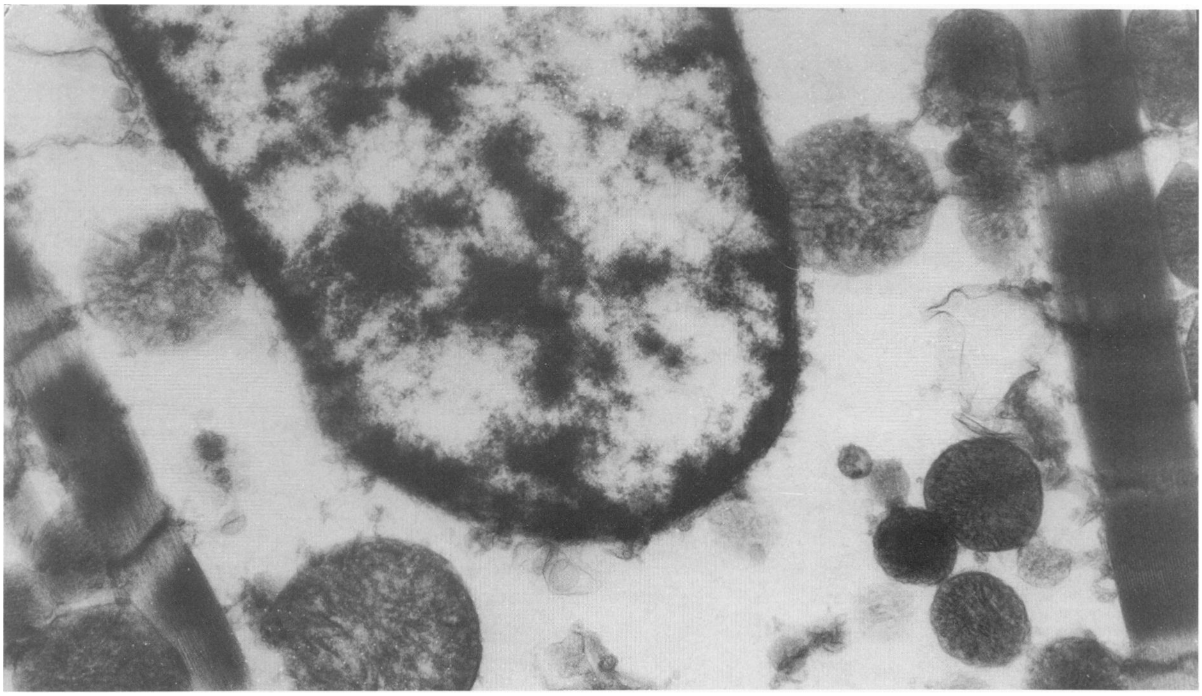

Figure 12—Seventy-five-minute anoxic swollen Group 1 heart. Anoxic cells contained empty-appearing perinuclear spaces with loss of both intermediate filament cables and microtubular profiles. Compare with Figure 6. (HVEM, 1 MeV, 0.5-μ section, ×12,000)

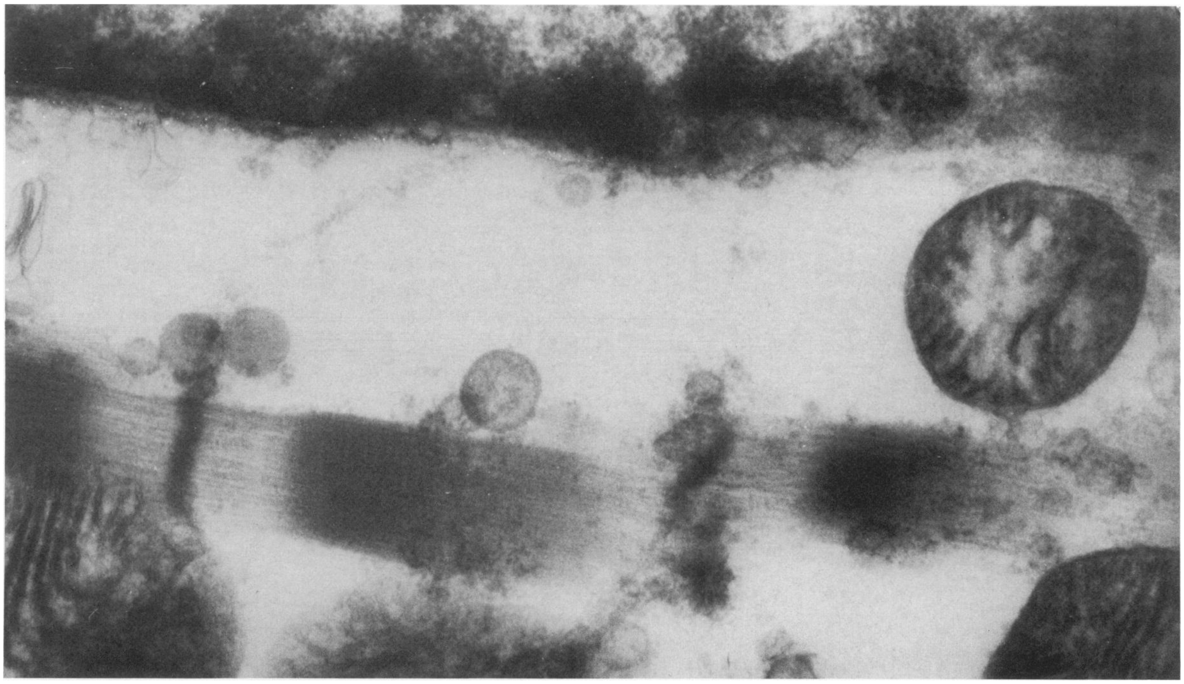

Figure 13—Seventy-five-minute anoxic Group 1 heart. In the perinuclear space of nearly every anoxic cell containing subsarcolemmal blebs, the Z-, M-, and A-band cables were broken and few or no microtubules were evident. Compare with Figure 7. (HVEM, 1 MeV, 0.5-μ section, $\times$28,000)

Figure 14—Ninety-minute anoxic Group 2 heart. Both swollen and nonswollen hearts showed contracture of sarcomeres with a retraction of sarcomeres away from the intercalated disk. Compare with Figure 4. (TEM, 60 keV, 900-Å section, $\times$9400)

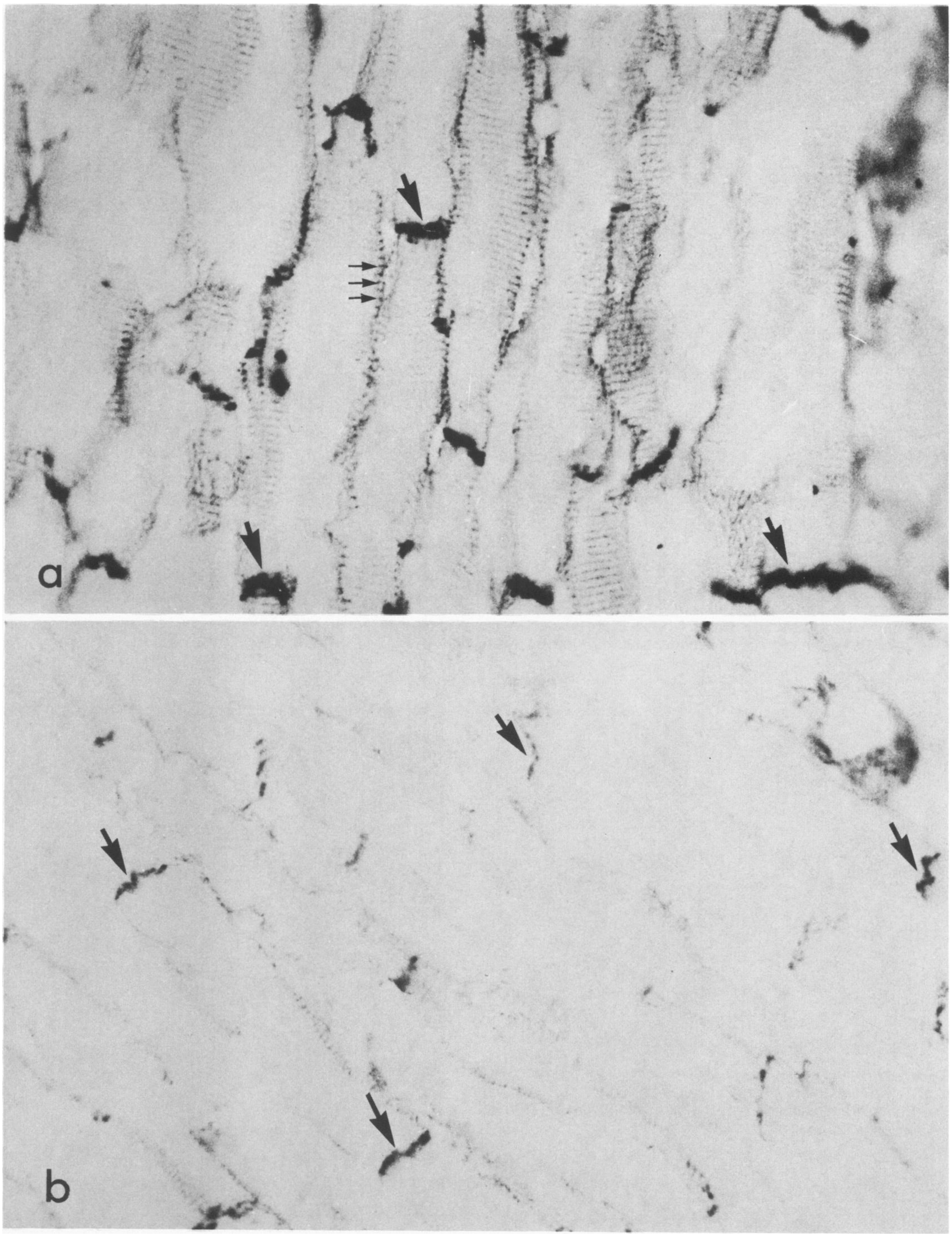

Figure 15—Figures 15–17 are direct negative prints from fluorescence-stained sections. Brightly staining areas appear dark on such prints. Figure 15a is of a control heart stained with anti-vinculin. Staining is localized to intercalated disks (*large arrows*) and focally along sarcolemmal membranes at intervals corresponding to lateral costamere junctions (*small arrows*). Figure 15b shows anti-vinculin staining of a 90-minute anoxic heart. There is extensive focal loss of lateral membrane staining and marked variation and decrease in staining of intercalated disks (*arrows*).

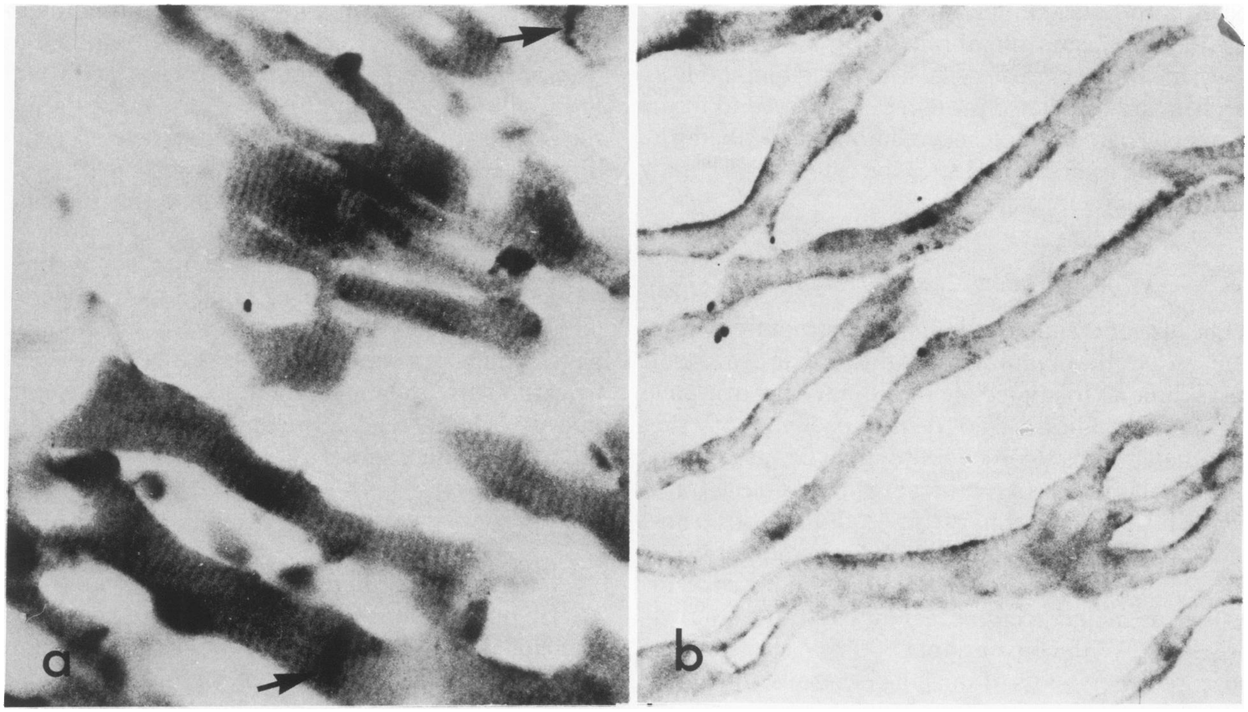

Figure 16—Control heart stained with anti-α-actinin. **a**—Intense staining along Z bands and at intercalated disks (*arrows*). There is a diffuse background cytoplasmic fluorescence. **b**—Loss of anti-α-actin staining from 90-minute anoxic hearts. A similar but less severe and more focal loss was observed at 45 minutes of anoxia.

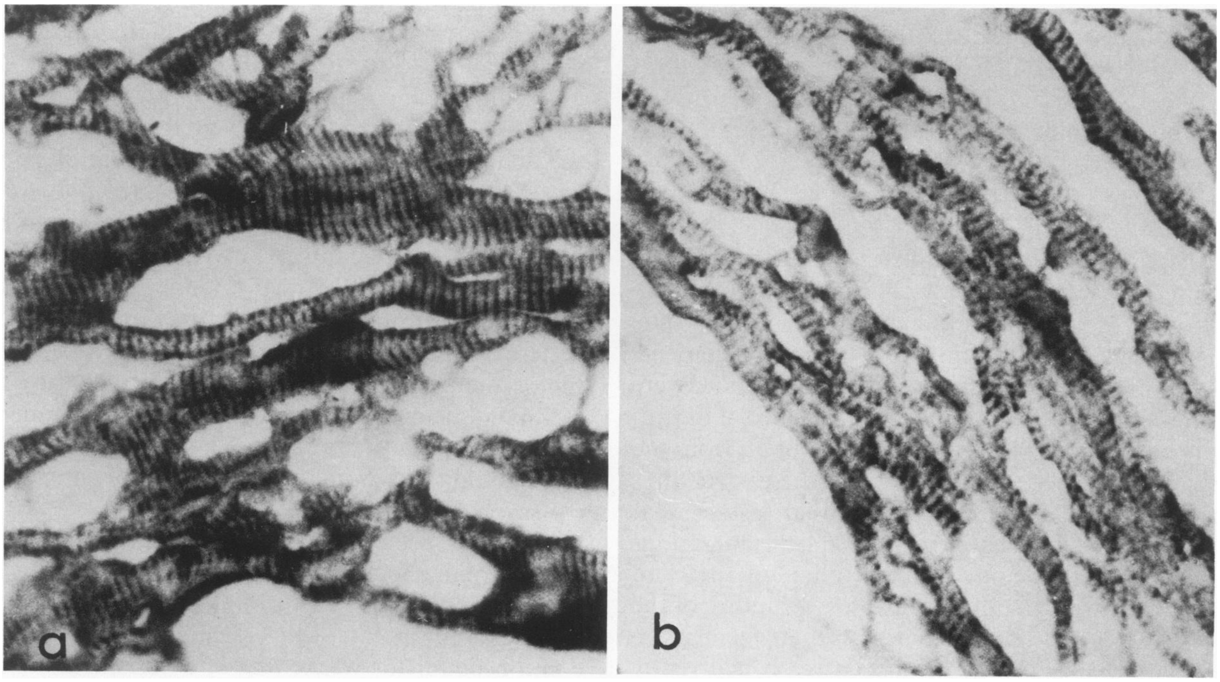

Figure 17—Anti-desmin staining of a control heart. **a**—a broad zone of staining along Z bands and diffuse cytoplasmic staining. No specific staining of intercalated disks was seen. **b**—In 90-minute anoxic hearts staining for desmin was less uniform than in controls but in most hearts remained of similar intensity. (×670)

spectrin and ankyrinlike proteins have been identified in the plasma membranes of a variety of cell types, including cardiac cells.[22] As in red blood cells,[8] spectrin and associated proteins are believed to form an integral part of the plasma membrane, stabilizing it and serving as anchoring sites for cytoskeletal[23] and transmembrane proteins.

Technical Considerations

Because of the highly filamentous structure of cardiac muscle, the organization of the cardiac cytoskeleton is difficult to appreciate in ordinary electron-microscopic studies. The dense packing of the sarcomeres tends to obscure the small filaments, and the long filamentous structures are impossible to trace for any distance in ordinary thin sections (50–100 nm thick). In the present study, the examination of swollen cells and the use of the HVEM microscope in examining thicker sections facilitated study of the cytoskeleton. With conventional 100 keV electron microscopes energy loss of imaging electrons and chromatic aberration of the lens limit the resolution to about one-tenth of the specimen thickness. Thus, a 0.2-μ section would be expected to yield a resolution of 200 Å; insufficient to study most cytoskeletal filaments. The HVEM operated at 1 MeV allows examination of up to 1-μ specimens with a limiting resolution approximating 1/200 of section thickness. Even with HVEM, useful section thickness is limited because the contrast of even strongly stained small objects progressively decreases with section thickness. The low contrast of the image causes HVEM pictures of thick sections to appear less distinct than those obtained from thin sections.

Anoxic Cytoskeletal Lesions

There was a number of different cellular cytoskeletal alterations apparent in swollen anoxic cells. Breakage of Z, M, and A band cable connections to the sarcolemma and retraction of cytoplasmic elements away from the raised sarcolemmal membrane were prominent lesions. Similar lesions occurred at the nuclear membrane. Microtubules were absent from the perinuclear space, suggesting that these structures may have depolymerized. Additional lesions that may possibly be related to cytoskeletal alterations were vesiculation of sarcoplasmic reticulum (SR) and the sarcolemmal membrane at sites of rupture of the blebs. The variety of lesions suggests that several pathologic mechanisms may have occurred concurrently to cause a widespread breakdown of the cytoskeletal system. The formation of subsarcolemmal blebs was closely associated with a loss of sarcolemmal membrane integrity. The magnitude of the myoglobin release from injured hearts correlated temporally with the swelling and quantitatively with the duration of the anoxic insult. In contrast to control or less severely injured cells, the cells possessing blebs did not respond to hypertonic fixatives by shrinking, which indicates that the ultrastructurally damaged cells were no longer capable of shrinking in response to a hyperosmotic solution. The loss of osmotic responsiveness is probably related to the rupture of the sarcolemmal blebs, which also permitted the leakage of myoglobin from the cells. The sum of these observations suggests that myoglobin release originated specifically from the cells in which subsarcolemmal blebs developed.

Immunofluorescence Studies

Changes in the immunofluorescence staining of anoxic myocardial cells were observed in hearts that had not been subjected to osmotic swelling before fixation. Therefore, rupture of sarcolemmal membranes with loss of a cytoplasmic pool of proteins is not a likely explanation for the loss of vinculin or α-actinin from their membrane or actin attachment sites. Bound vinculin is known to be in exchange equilibrium with a soluble cytoplasmic pool, and loss of this soluble pool from cells with ruptured blebs could cause a loss of membrane-bound vinculin. Evidence that this can occur came from our observations that the loss of staining for vinculin and α-actinin was indeed much more severe in hearts that had been swollen prior to fixation (data not shown). The decrease in staining for vinculin and α-actinin in nonswollen hearts is therefore an event that occurs before the actual formation of blebs and rupture of sarcolemmal membranes. Our results confirm the results of Steenbergen et al,[25] who also found loss of staining for vinculin in ischemic dog myocardium.

In cardiac muscle, vinculin is localized to costamere–sarcolemmal membrane junctions and fascia adherens junctions of intercalated disks. Vinculin is a major membrane-associated component of the attachment plaques of stress fibers and other membrane-actin attachment sites. Therefore, loss of vinculin could contribute to detachments of actin from the sarcolemma.

Recently spectrin has been localized to sarcolemmal membranes at Z- and M-band levels and has itself been shown capable of forming lateral associations with desmin.[22,23] Although loss of vinculin or spectrin could account for weakening of cytoskeletal–membrane attachments, too little is known of the biophysical nature of these associations in cardiac cells to warrant further speculation. Similar arguments apply to the binding of decreased α-actinin fluorescence. It is, however, possible to speculate that loss of α-actinin from Z bands and fascia adherens junctions contributes to Z-band lysis and separations of sarcomeres from intercalated disks. In contrast to the other proteins, desmin appeared more stable during anoxic perfusion and showed relatively minor changes until late in severely altered cells.

Thus, even though we were able to clearly demonstrate a decrease or loss of immunofluorescence staining of vinculin and α-actinin, the significance of the loss of staining is uncertain. Decreased staining could occur as a result of a reversible blockage of antibody binding sites, changes in the tertiary structure of the molecule, or redistribution of the protein, as well as loss or proteolytic degradation of the proteins. Until more quantitative assessments are available, the immunofluorescence results must receive cautious interpretation.

Cell Swelling and the Cytoskeleton

In vitro preparations of isolated calcium-tolerant isolated myocytes do not swell in an ideal manner when exposed to media of varying osmotic strength.[24] The extensive cytoskeletal network has been proposed to account for this significant passive resistance of myocardial cells to hypotonic swelling.[24] Control myocardium can be subjected to osmotic swelling without demonstrable sarcolemmal damage. Slices of normoxic dog myocardium can be exposed to 60-mOsm/l media without sustaining an increase in inulin-diffusible space.[6] We have exposed normoxic perfused rat hearts to 150-mOsm/l perfusion media without observing significant creatine kinase or myoglobin release.[7] In the present study, the ability of oxygenated cells to withstand severe osmotic stresses was shown by both the absence of myoglobin release and the ability of the previously swollen cells to shrink and to maintain nearly normal ultrastructure when exposed to hypertonic fixative solutions.

Speculations on Irreversible Injury

The lesions that occur in the organization of the cardiac cell cytoskeletons during anoxic perfusions could contribute to the development of irreversible myocardial cell injury. The cytoskeletal apparatus may play an important role in maintaining myocyte viability. The results of this study allow the postulation of a sequence of events that could cause irreversible myocardial injury. Energy depletion during the anoxic or ischemic injury leads initially to an alteration of the cells' ability to maintain cytoskeletal integrity. The alterations of cytoskeletal-related proteins cause the cells to become fragile so that they can no longer withstand normal physical stresses. It is at this point that we believe irreversible injury occurs. When such cells are swollen either by hypotonic swelling, as in the present experiment, or, as Jennings' group[6] has suggested, reperfusion of hypertonic ischemic cells with isotonic blood, the physical stresses of swelling rupture the weakened sarcolemmal–cytoskeletal attachments, allowing formation of subsarcolemmal blebs. The loss of cytoskeletal support for the plasma membranes overlying the blebs predisposes to rupture of the plasma membranes and cell death.

However, simple swelling is not an adequate explanation for the massive cell injury and enzyme release seen when anoxic hearts are reoxygenated. In the anoxia–reoxygenation model of cell injury, continuous perfusion allows washout of lactate and other small molecules which are the major contributors to the osmotic load in ischemia.[5,6] We have shown that prevention of contracture in reoxygenated cells by a variety of mechanisms will prevent reoxygenation enzyme release.[3] Elz and Naylor[20] have recently shown that BDM, a compound that specifically inhibits actin–myosin interactions,[31] also prevents both contracture and enzyme release. Furthermore, it has been shown that the oxygen paradox does not occur in isolated anoxic myocyte preparations that have been reoxygenated.[32,33] These observations, coupled with our previous observations that contracture and enzyme release are closely associated events, lead us to hypothesize that the mechanical stresses imposed on cells with weakened cytoskeletal attachments may be the precipitating cause of sarcolemmal membrane rupture during the oxygen paradox.[7] The formation of contraction bands may cause focally severe physical forces to be exerted on intermediate filament cables, ultimately leading to rupture of cytoskeletal-sarcolemmal attachments and formation of subsarcolemmal blebs. Mechanical deformation of these blebs could then lead to sarcolemmal membrane rupture and cell death.

Mechanism of Cytoskeletal Injury

There are several possible mechanisms which could produce cytoskeletal damage, the actual causes are at this time unknown. Myocardial cells contain a variety of calcium-dependent cytoplasmic proteases called calcium-activated neutral proteases (CANP) or calpains.[26–28] Some of these proteases have been shown to hydrolyse cytoskeletal proteins. Activation of these proteases is one possible mechanism of injury. Other cytoskeletal proteins may be phosphorylation-dependent.[8] Therefore, it is possible that the severe loss of energy and phosphorylation potential that occurs during anoxia and ischemia may lead to weakening or destruction of one or many phosphorylation-dependent cytoskeletal proteins crucial to maintaining cellular integrity. Future studies will be required to determine the exact mechanism(s) of anoxic cytoskeletal damage.

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